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Table of Contents

Role of the Stem Cell Niche in Hormone-induced Tumorigenesis in Fetal Mouse Mammary Epithelium

Abstract

Stem cells possess properties that compel the tissue to devise some method to contain and husband them. They are mobile, environmentally highly responsive, self renewing and pluripotent. A structure specialized to contain and regulate stem cell activity has been structurally and molecularly described in Drosophila and some mammalian tissues. The structure, the stem cell niche, functions to 1) shield the stem cell from the burden of incoming hormone and growth factor signals present in the surrounding tissue, 2) supply, interpret, and regulate specific signals to modulate tissue growth by directing stem cell renewal and maturation, 3) prevent stem cells from wandering through the tissue and producing new cells inappropriately, 4) prevent stem cells from leaving the tissue inappropriately and potentially colonizing other tissues. Because of it potential in preventing tumorigenesis we turned our attention to it description in situ in mouse mammary epithelium. We are developing immunostaining staining protocols for molecules shown to be involved in niche signaling in drosophila and mammalian gut and have been successful with P63, Notch-1 and Oct-4. Our results to date suggest that the stem cell niche in the mammary gland may exist in an extended hierarchy or layered structures rather than the simple organization seen in drosophila gonads.

Introduction.

The emerging view of cancer is that it results from the hijacking of developmental signaling pathways in adult tissues to initiate inappropriate growth. There is also evidence that excess hormonal exposure during critical developmental windows may make some tissues, such as breast, susceptible to such hijacking of developmental pathways (Hilakivi-Clarke, 2002) in later life. Such exposure is believed to affect the primary stem cells of the newly forming organ. Although a great deal of work addresses the biology of breast cancer, our rudimentary understanding of the basic biology of the mammary gland is retarding our ability to gain not only critical information concerning tumorigenesis, but also the development of better paradigms for developing new research approaches. Since the biology of the mammary gland begins with it's stem cells and their health maintenance, it is critical to understand how normal breast stem cell proliferation is regulated for lactation and maintenance and how it can be disrupted to produce breast cancer.

The putative stem cells of the mammary epithelium are structurally undifferentiated cells (Chepko and Smith, 1997) that are sequestered in the epithelium by closely associated luminal and myoepithelial cells with specialized cytoplasmic extensions that separate them from the basement membrane (Chepko and Dickson, 2003). These luminal cells also close over the stem cells apically, sequestering them from the lumen as well. This forms a closed microenvironment or "niche" that physically limits exposure to incoming stimuli. Coated vesicles seen in putative stem cells suggest that they receive paracrine signals from their neighbors (Chepko and Dickson, 2003). At the light microscope level these putative niche cells are much thinner and more deeply staining than other luminal cells (Fig. 1). We believe that they may define the stem cell niche and act to regulate signaling to the stem cells that reside within their enclosure.

To determine if this may be so we have proposed to develop staining protocols for receptors that in other stem cell systems have specificity for niche cells, and that demonstrate high interspecies molecular conservation (Spradling et al., 2001;Xie and Spradling, 2000;Ohlstein et al., 2004). We also proposed to test putative stem cell markers in mammary epithelium in an attempt to reach consensus between morphological and functional definitions of cell types and aid in the definition of the niche cells. Finally we would use in utero stimulation with estrogen to determine if either the stem cells or the niche cells were affected in the adult. Cell-specific immunostaining has been difficult in mammalian systems because of the high background caused by the combined confounding factors of non-specific interactions with extracellular matrix proteins and lipoproteins and the low specific protein expression in the cells of interest. For the development of the protocols we used archival normal mouse tissue from previous studies so that we would not use up experimental tissues just for developing staining protocols. The protocol development proved so time consuming, that we have not treated any animals with estrogen and therefore have progressed only through the main part of our first objective (see below). *We have successfully developed protocols for P63, Oct-4, and Notch-1. Bmi-1 is still in development, and Sca-1, and ABCG2 (Bcrp-1) are expressed at such low levels they cannot be used for immunostaining.*

Body

Rationale/Purpose as Stated in Proposal: Some breast cancers may derive from high *in utero* estrogenic influences that permanently alter signaling in the adult stem cell niche. This changes the ratio of proliferative to differentiated cells leading to the epithelial disorganization seen in cancer.

Objectives as Stated in Proposal: There are two basic objectives to this proposal. We will use morphological assessment and the expression of stem cell markers to **1)** structurally and molecularly characterize the mammary stem cell niche *in situ* and **2)** determine how these characteristics are changed by *in utero* estrogenic influences. These aims will be studied using FVB mice that will be exposed to estradiol *in utero* through a pregnant dam. *This will produce the hypothesis that the mechanism of estrogen carcinogenesis acts to change stem cell kinetics,* which can then be addressed utilizing mammary epithelial transplantation techniques.

Materials and Methods.

Normal FVB mouse mammary gland, intestine, ovary, epidermis and hair follicle plus MMTV-c-myc and bitransgenic c-myc-TGFα mouse mammary tumors, and liver, fixed at 4°C for 6 hours in 4% paraformaldehyde in 0.5M PBS at pH 7.2.
Immunostaining was performed at antibody dilutions ranging from 1:10 to 1:300 at 4°C overnight or at room temp for 30-60 minutes. These tests were performed using the Vectastain Elite, DAKO ARK, CSA, Envision, or Envision Plus Kits and the following antibodies: Sca-1 MAB 1226 (R&D Systems), ABCG2 MAB 995 (R&D Systems), Oct-4 sc-5279 (Santa Cruz,) P63 ab3239 (abcam), Notch-1 ab1 MS-1339-P1 (NeoMarkers), PR-AT 4:14 anti-Progesterone receptor (PR). The monoclonal antibody PR-AT 4.14 against peptides 533-547 of human progesterone receptor was the gift of Dr. Abdulmaged Traish, and recognizes PR of mouse uterus (Traish and Wotiz, 1990).

Key research accomplishments:

Found best results with DAKO's Envision Plus kit for all antibodies.
  ➢ Achieved positive staining of P63
  ➢ Achieved positive staining of Oct-4

> Although none of these molecules is a marker for stem or progenitor cells, they may be useful as tools to define transit amplifying cells, the stem cell niche, and it's function.
> Sca-1, ABCG2 are present at such low expression they cannot be used for paraffin sections.
> Bmi-1 results not conclusive yet, but the positive control (ovary) has produced good positive results.

Reportable Outcomes.

Archival tissue from previous experiments was used to work out the stains. Protocol development has been difficult due to very low expression of the proteins of interest, and the problem of high nonspecific staining with some antibodies. Protocol development for immunostaining has consumed all of the allotted time. P63, Oct-4 and Bmi-1 were thought at the time we wrote the grant to be proteins with possible stem cell specific expression. P63 has recently been shown to specify stratification for all stratified epithelia (Koster et al., 2004), and to be a specific marker for myoepithelial cells in dog mammary epithelium (Gama et al., 2003).

P63 Our results with this antibody showed the same for mouse mammary epithelium (Fig. 2) and also that it stains most of the cells in epidermis and hair follicle (Fig. 3). This result is consistent with the results of Koster et al (Koster et al., 2004) that demonstrate its involvement in epithelial stratification. Since it is present in adult tissue it may play a role in maintenance of stratification, and therefore an important role in niche cell function. P63 positive cells were rare in c-myc and c-myc/TGFα bitransgenic mammary tumors.

Oct-4 A subset of all cell types in mammary epithelium was positive for Oct-4, and roughly 50% of all mammary epithelial cells were Oct-4 positive (Fig. 2). This was also true for epidermis and hair follicle (Fig. 3). Stem cells are believed to be a rare cell type in all tissues, and because so many cells are positive for Oct-4 it cannot be used to demonstrate stem cell localization *in situ*. However, it may be useful as an indicator of transit amplifying (progenitor) cells. I epidermis sebaceous cells were Oct-4 positive whilst they were negative for P63.
Notch-1 Notch proteins are known to act in cell fate determination in many tissues and are acciated with stem cell niche function in Drosophila ovary (Xie and Spradling, 2000), and in mammalian cancers (Weng and Aster, 2004). Our staining opposite to that seen with notch-4 in mouse mammary gland: the large ducts are negative and the lobular tissue and small ducts are positive.
Progesterone Receptor Staining with anti-progesterone receptor revealed that putative progenitor cells in putative niches were positive for progesterone receptor whilst the niche-like cells were negative. However, a subset of all cell types except myoepithelial cells and perhaps, niche-like cells, were positive for PR. Myoepithelial cells were uniformly negative for PR.

Conclusions Although in simple systems such as drosophila the stem cell niche consists of a few niche cells and one stem cell and possibly a few daughters, the stem cell niche in a complex, hormone responsive tissue such as mammary gland may actually exist in tiers or layers. Our results to date suggest this possibility. The limit of P63 expression to the myoepithelial cells and their location in the epithelium exclusively at the basement membrane and below the lumen position them for an interpretive and instructive role vis a vis signaling. Also the negative staining, but specialized positioning of putative niche cells relative to putative stem/progenitor cells is suggestive of this kind of role. For these reasons we are hopeful about continuing to pursue the staining still on the agenda and also testing the newly available antibody to integrin 6α (CD49f, Stemcell Technologies) to try define cell type roles in this complex epithelium.

Cd49f has been developed for mouse mammary stem cell isolation by FACS (Stemcell Technologies).

Future: As stated in the sf298 we have applied for a no cost extension to pursue the rest of our agenda. We plan to continue to develop a staining protocol for Bmi-1 and add CD49f (Stemcell Technologies). When the protocols are complete we will perform the estrogen study outlined in our second objective.

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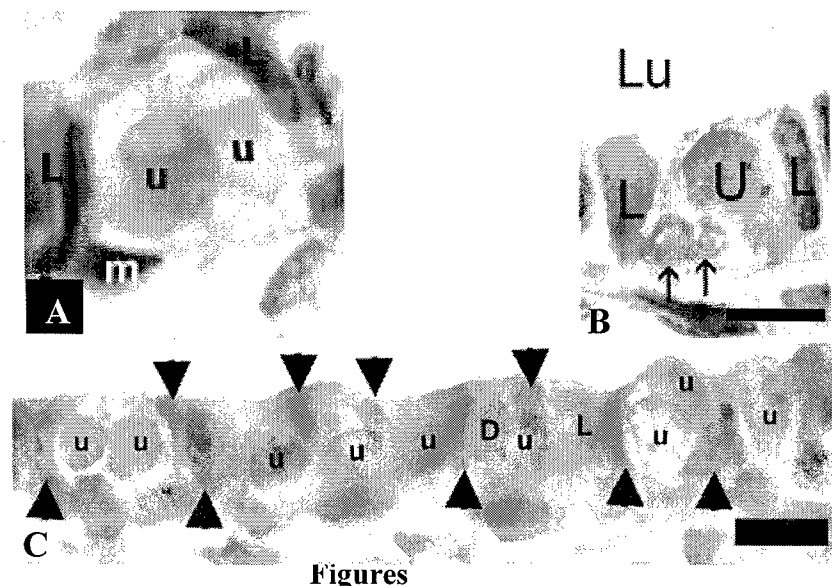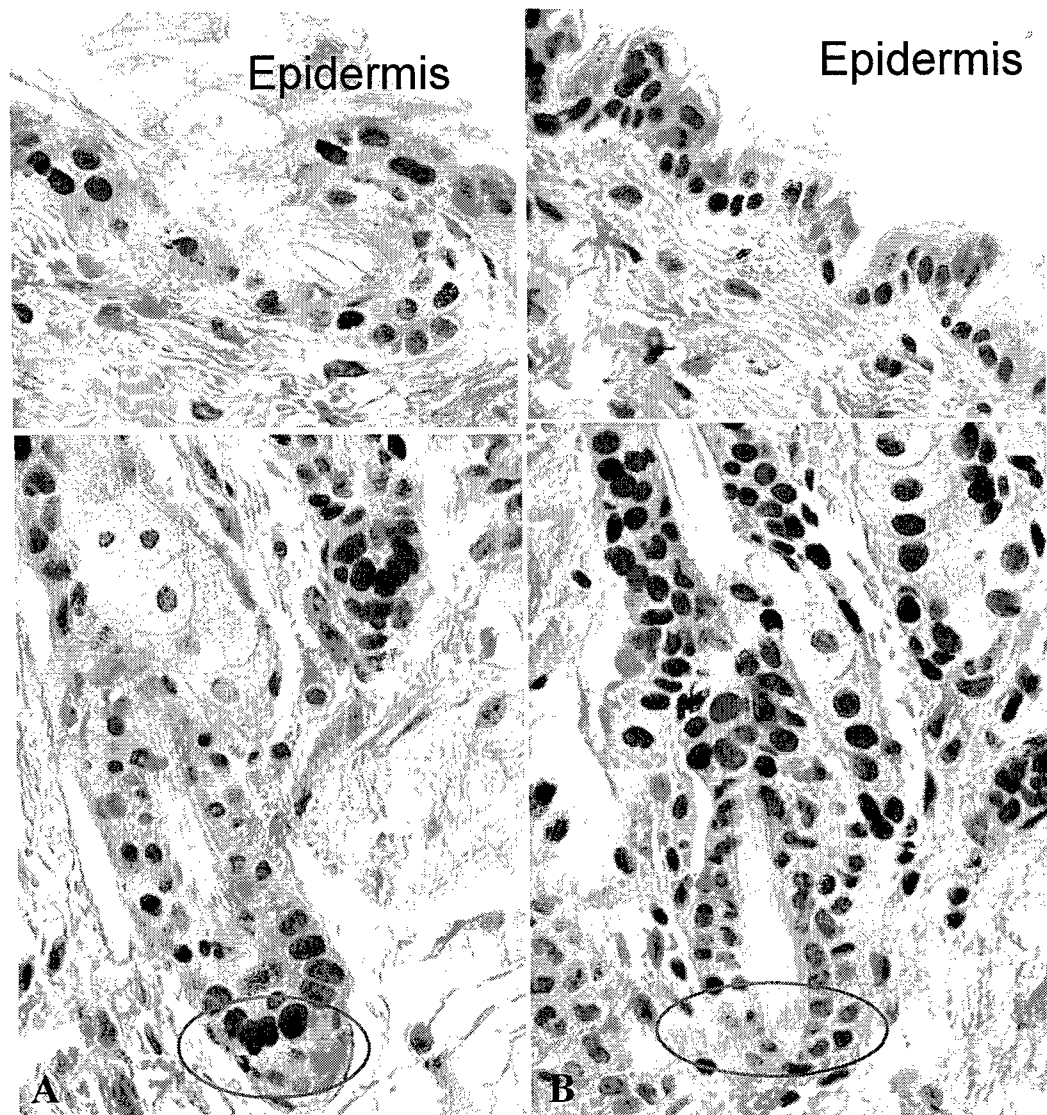
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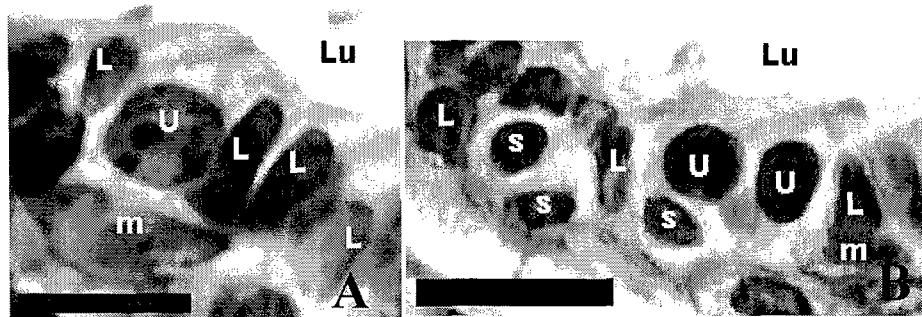
Figure 1. Stem Cell Niches in mammary ductal epithelium of nulliparous FVB mouse. A. Two putative progenitor cells (u) flanked by putative niche cells (L). B. Two Putative niche cells flanking a putative progenitor cell (U) and two putative stem cells (arrows). C. A longitudinal section through a mammary duct showing a row of repeating putative niches
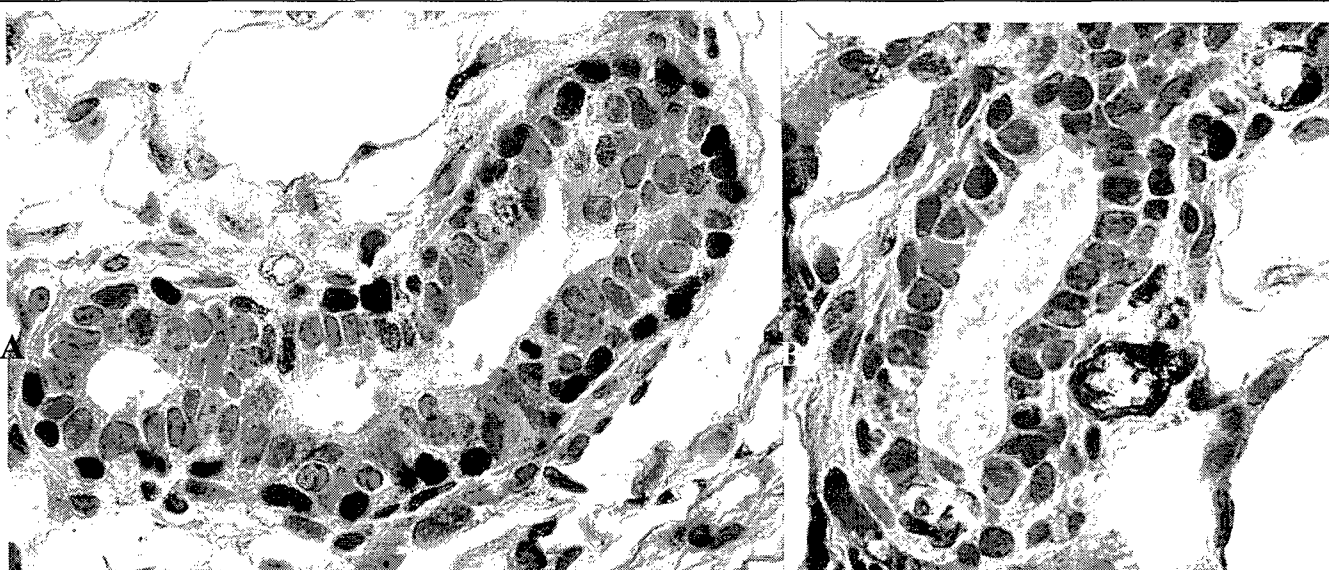

Figure 2. Comparison of P63 and Oct-4 localization in mouse mammary ducts. Anti-P63 (A) and Anti- Oct-4 (B). In (A) all myoepithelial cells are positive for P63 (brown) and all other cell types are negative (blue). Oct-4 (B) stains subsets of all cell types (brown), but many cells are negative (blue).

Figure 3. Comparison of P63 (A) and Oct-4 (B) in epidermis and hair follicle (lower panels). Although many cells are positive none are stem cells because epidermal stem cells are located in the encircled region and in the "bulge"

Figure 4. Mammary ductal epithelium of nulliparous 35 day old mouse stimulated with progesterone for 9 days. Anti-progesterone receptor (brown) is present in putative progenitor cells (U) and a subset of putative stem cells (s) but absent in niche-like cells (L). m; myoepithelial
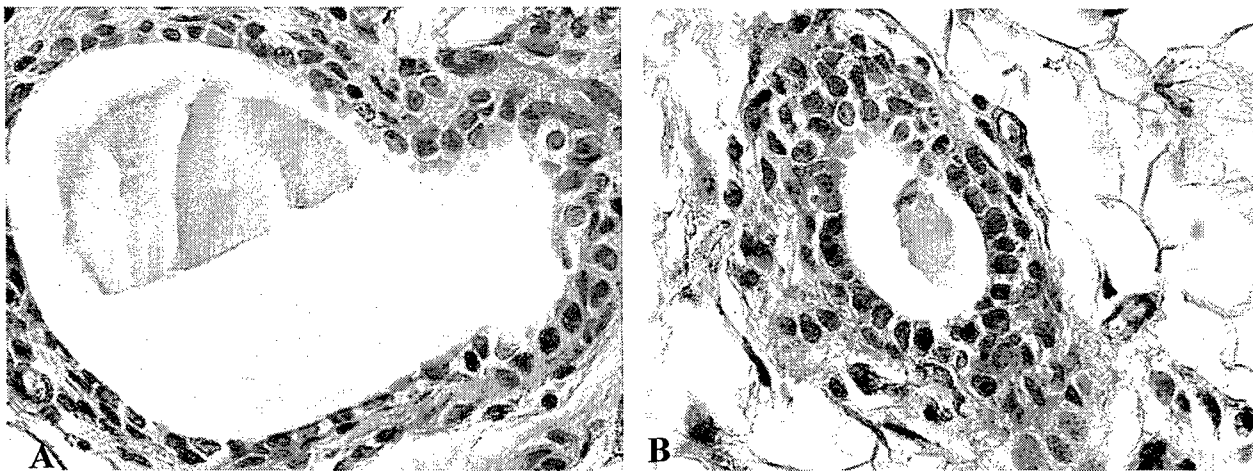
Figure 5. Anti- Notch-1 staining. **A.** Large duct epithelium showing negative staining for Notch-1. **B.** Small duct epithelium